

# Dnmt2-dependent methylomes lack defined DNA methylation patterns

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Contributed by Gregory J. Hannon, April 9, 2013 (sent for review February 15, 2013)

Several organisms have retained *methyltransferase 2 (Dnmt2)* as their only candidate DNA methyltransferase gene. However, information about Dnmt2-dependent methylation patterns has been limited to a few isolated loci and the results have been discussed controversially. In addition, recent studies have shown that *Dnmt2* functions as a tRNA methyltransferase, which raised the possibility that *Dnmt2*-only genomes might be unmethylated. We have now used whole-genome bisulfite sequencing to analyze the methylomes of *Dnmt2*-only organisms at single-base resolution. Our results show that the genomes of *Schistosoma mansoni* and *Drosophila melanogaster* lack detectable DNA methylation patterns. Residual unconverted cytosine residues shared many attributes with bisulfite deamination artifacts and were observed at comparable levels in *Dnmt2*-deficient flies. Furthermore, genetically modified *Dnmt2*-only mouse embryonic stem cells lost the DNA methylation patterns found in wild-type cells. Our results thus uncover fundamental differences among animal methylomes and suggest that DNA methylation is dispensable for a considerable number of eukaryotic organisms.

RNA methylation | epigenetics

DNA methylation is an epigenetic modification with important functions in cellular differentiation, organismal development, and human disease (1, 2). DNA methylation is widely conserved in the animal and plant kingdoms and established and maintained by a family of enzymes termed DNA methyltransferases (3, 4). In animals, DNA methyltransferase 3 (*Dnmt3*) enzymes act as de novo methyltransferases that establish DNA methylation patterns, most prominently during early stages of development. Established methylation patterns are then maintained by DNA methyltransferase 1 (*Dnmt1*) enzymes, which copy methylation marks from methylated CpG dinucleotides on the parental DNA strand to complementary CpG dinucleotides on the daughter strand.

DNA methyltransferase 2 (*Dnmt2*), which is also known as tRNA aspartic acid methyltransferase 1 (*Trdm1*), is the second member of the DNA methyltransferase family and shows strong sequence conservation to the catalytic motifs of established DNA methyltransferases (5–7). However, the actual DNA methyltransferase activity of *Dnmt2* was found to be substantially weaker than for other DNA methyltransferases (8, 9). It was later shown that *Dnmt2* has a robust methyltransferase activity toward cytosine 38 in the anticodon loop of tRNA<sup>Asp</sup> and other tRNAs (9, 10) and that the enzyme uses the conserved DNA methyltransferase mechanism to methylate tRNA (11). In agreement with this notion, several independent phylogenetic analyses have suggested that *Dnmt2* is an ancient DNA methyltransferase that has switched its substrate specificity from DNA to tRNA (12–14).

The number of *Dnmt* genes can vary greatly between genomes, and various organisms have been shown to encode different sets of *Dnmt* enzymes (15). Mammalian genomes encode one *Dnmt1* gene and three paralogs of *Dnmt3*. This contrasts, for example, with the genome of the parasitic wasp *Nasonia vitripennis*, which

encodes three paralogs of *Dnmt1* and a single *Dnmt3* homolog (16). These variations have been interpreted to reflect multiple versions of a tool kit for phenotypic adaptation (17). During evolution, parts of this tool kit could have been contracted or expanded to facilitate specific requirements for genome regulation (17). In this context, *Dnmt2* has been shown to provide a link between environmental cues, such as stress and nutrition signals, to tRNA stability and the regulation of protein synthesis (10, 18, 19).

Interestingly, there is a diverse group of animal species that has retained *Dnmt2* as their only candidate DNA methyltransferase. This group includes several highly relevant model organisms, such as *Schizosaccharomyces pombe*, *Dictyostelium discoideum*, *Entamoeba histolytica*, *Schistosoma mansoni*, and *Drosophila melanogaster*. Global DNA methylation levels in these *Dnmt2*-only systems have been found to be very low and have often been discussed controversially (20–22). More recently, however, two prominent studies have provided support for a biologically important function of *Dnmt2*-dependent DNA methylation. For example, it has been suggested that *Dnmt2*-dependent DNA methylation regulates oviposition in *S. mansoni* (23), the causative agent of bilharziosis. In addition, *Dnmt2*-dependent methylation of transposons has been linked to genome stability in *D. melanogaster* (24). These results have necessitated a more detailed analysis of genome methylation patterns in *Schistosoma* and *Drosophila*.

Over the past few years, whole-genome bisulfite sequencing has been established as a method to characterize genome-wide DNA methylation patterns at single-base resolution (25). This method has been successfully used to characterize the methylomes of various animal organisms that are known to establish and maintain their methylation patterns by *Dnmt1* and/or *Dnmt3* enzymes. The results revealed a certain degree of diversity among animal methylomes, but also identified a number of conserved features, which include the specificity for CpG dinucleotides and an enrichment of methylation in defined genetic elements (26, 27). We have now used whole-genome bisulfite sequencing for an unbiased characterization of the *Schistosoma* and *Drosophila* methylomes. Our results fail to reveal any evidence for defined DNA

Author contributions: G.R., P.M.G., G.J.H., and F.L. designed research; G.R., P.M.G., N.O., and M.S. performed research; M.R.F. and M.R. contributed new reagents/analytic tools; G.R., P.M.G., N.O., M.S., W.R., G.J.H., and F.L. analyzed data; and G.R., G.J.H., and F.L. wrote the paper.

The authors declare no conflict of interest.

Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, [www.ncbi.nlm.nih.gov/geo](http://www.ncbi.nlm.nih.gov/geo) (accession nos. GSE39996, GSE39997, and GSE42170).

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This article contains supporting information online at [www.pnas.org/lookup/suppl/doi:10.1073/pnas.1306723110/-DCSupplemental](http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.1306723110/-DCSupplemental).

methylation patterns in these organisms and thus uncover fundamental differences between Dnmt1/3-dependent and Dnmt2-dependent methylomes.

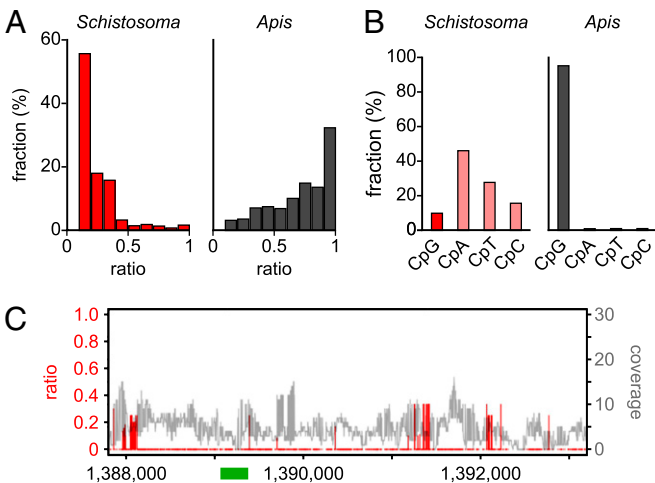
### Results

**Whole-Genome Bisulfite Sequencing of Three Independent Dnmt2-Only Models.** We investigated Dnmt2-dependent DNA methylation in three independent models: (i) *S. mansoni*, (ii) *D. melanogaster*, and (iii) a triple knockout (TKO) mouse embryonic stem cell line, which is deficient for Dnmt1, Dnmt3a, and Dnmt3b but has retained an intact *Dnmt2* gene (28). All samples were obtained from the same strains and developmental stages that were used for previous studies (Table 1). Furthermore, Dnmt2 activity was confirmed by methylation analysis of tRNA<sup>Asp</sup>, which showed high levels of methylation for the established C38 target site in all three samples (Fig. S1).

We then used whole-genome bisulfite sequencing to comprehensively analyze genomic DNA methylation patterns in all three models. After bisulfite deamination, DNA libraries were prepared and subjected to paired-end Illumina sequencing. Read pairs were subsequently mapped to the corresponding reference genomes using BSMAP 2.0 (29). This resulted in average strand-specific genome coverages of 13× for *Schistosoma*, 32× for *Drosophila*, and 1× for TKO cells (Table 1). The conversion rate of mapped cytosine residues was >98.0% in all cases (Table 1), which suggested that bisulfite deamination had been efficient and that the sequence information could be used for methylation analysis.

**Methylome of *S. mansoni*.** As an initial step toward a comprehensive analysis of the *Schistosoma* methylome, nonconversion ratios were determined for all cytosine residues with a sequence coverage greater than three. This revealed that 97% of the cytosines were completely converted (ratio <0.1). When the remaining cytosines were distributed into bins with increasing nonconversion ratios, the majority (93%) showed ratios <0.5, whereas only a minor fraction (7%) had ratios >0.5 (Fig. 1A). This distribution strongly contrasts the results from honey bees, which have an established Dnmt1/3-dependent methylome with very low levels of DNA methylation (30). Here, only 21% of all not completely converted cytosines showed a nonconversion (methylation) ratio <0.5, whereas 79% had a ratio of >0.5 (Fig. 1A). These results suggest that the *Schistosoma* genome contains either extremely low levels or no DNA methylation at all.

A conserved feature of all known animal methylomes is their high degree of CpG-specificity (26, 27), which is related to the stable transmission of symmetric CpG methylation marks by maintenance methylation mechanisms. Therefore, we also determined the dinucleotide sequence context of nonconverted cytosines in our *Schistosoma* dataset and could not detect any evidence for CpG specificity (Fig. 1B). In contrast, nonconverted cytosines from the honey bee methylome dataset were strongly (>95%) enriched for CpG dinucleotides (Fig. 1B). Finally, we also used our data for a detailed analysis of the *Schistosoma forkhead* gene that had previously been reported to be methylated in a Dnmt2-



**Fig. 1.** Characterization of the *Schistosoma mansoni* methylome. (A) Average methylation levels were determined for all cytosine residues with a methylation ratio >0.1 and then distributed into bins with increasing methylation ratios (red bars). For comparison, the corresponding data are also shown for honey bee worker brains (gray bars), an established Dnmt1/3-dependent methylome with a very low DNA methylation level (30). (B) Dinucleotide sequence contexts of unconverted cytosines in *Schistosoma* (red) and in honey bees (gray). (C) Position-specific nonconversion ratios (red) and coverages (gray) of the *Schistosoma forkhead* gene. The specific region previously reported to be methylated (23) is indicated as a green bar. Sequence position numbers refer to GenBank accession JF781495.

dependent manner (23). Our results failed to reveal any evidence for DNA methylation at the previously analyzed region (Fig. 1C). We did, however, observe dense clusters of incompletely converted cytosines outside of this region (Fig. 1C), preferentially in sequences with low complexity. Similar patterns have not been described in any of the published methylomes, but correspond to known characteristics of bisulfite deamination artifacts (31). Taken together, these results strongly suggest that the *Schistosoma* genome is unmethylated.

**Methylome of *D. melanogaster*.** To characterize the methylome of a second Dnmt2-only organism, we obtained genome-wide methylation profiles from *D. melanogaster* embryos. A previous phylogenetic analysis of DNA methylation patterns had already suggested that DNA from *Drosophila* embryos is unmethylated (27). However, the data were obtained from an unspecified strain and the sequencing coverage was comparably low. To further characterize the *Drosophila* methylome, we therefore obtained an independent methylation profile from 0- to 2-h-old wildtype (*w*<sup>1118</sup>) embryos. This is the same strain and developmental stage that was used for a previous analysis describing DNA methylation of *Invader4* elements (24). As a reference, 1% of human sperm DNA was spiked into the *Drosophila* sample before bisulfite

**Table 1. Sequencing data**

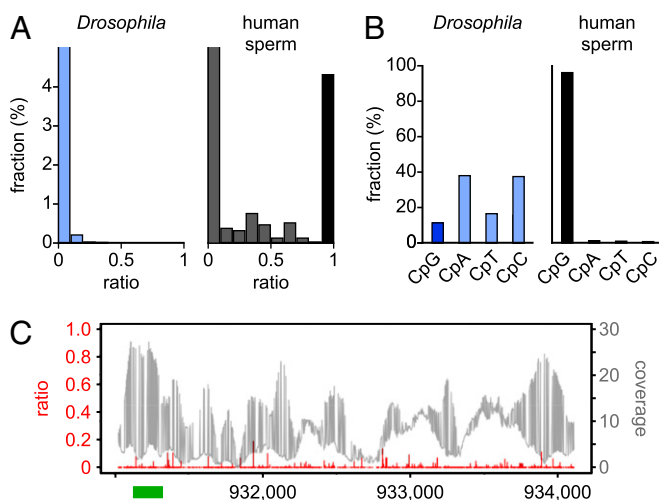
Sample	Comment	No. of reads (pairs)	Mapping efficiency, %	Coverage	Conversion, %
<i>Schistosoma</i>	Mixed adult Puerto Rican	254,347,320	73	13.4×	99.02
<i>Drosophila</i>	<i>w</i> <sup>1118</sup> 0–2 h embryos	174,821,591	57	32.0×	99.75
TKO	Mouse TKO ES cells	43,583,131	74	0.8×	98.22
PCR fragment	Spiked into TKO	1,593	ND	ND	98.38
Human sperm DNA	Spiked into <i>Drosophila</i>	1,637,070	ND	ND	99.71
<i>Drosophila</i> Dnmt2 <sup>−/−</sup>	Dnmt2 <sup>149</sup> 0- to 2-h embryos	129,094,743	52	23.6×	99.42

Sequencing coverages are indicated per strand. Conversion rates were calculated as the average conversion ratio of all cytosine residues that were covered by the dataset. For human sperm DNA, the conversion rate was calculated as the average conversion ratio of all non-CpG dinucleotides that were covered by the dataset. ND, not determined.

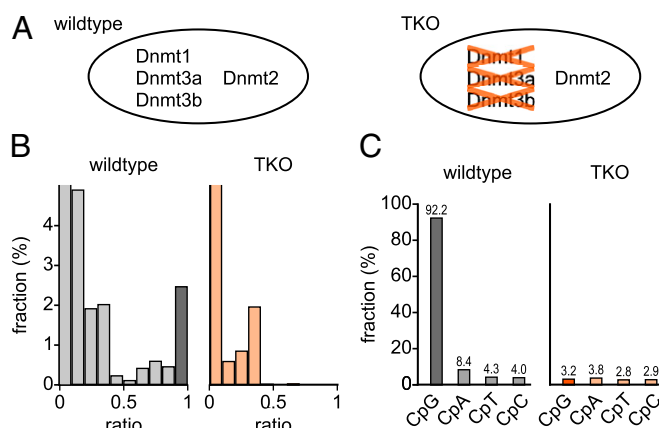
conversion. Human sperm DNA is known to be highly methylated (32) and the spiked-in DNA sample thus served as an important internal control.

A detailed analysis of the *Drosophila* data showed that the vast majority (99.7%) of cytosine residues appeared completely unmethylated (ratio <0.1), whereas only 0.003% showed a non-conversion ratio >0.5 (Fig. 2A). This distribution was substantially different for the spiked-in human sperm DNA, which showed complete methylation (ratio >0.9) for 4.3% of the cytosine residues that were analyzed (Fig. 2A). Pronounced differences between the *Drosophila* and the control sample were also detectable for the dinucleotide sequence context of non-converted cytosine residues. In the *Drosophila* dataset, only 11% of the nonconverted cytosine residues were found in CpG dinucleotides (Fig. 2B). This distribution strongly contrasted the control sample, which showed a high degree (98%) of CpG specificity (Fig. 2B). Finally, we also used our data for a detailed analysis of *Drosophila* *Invader4* elements, which have previously been reported to be methylated in a Dnmt2-dependent manner (24). Our results failed to reveal any evidence for DNA methylation at the previously analyzed region and at other *Invader4* sequences (Fig. 2C). Together, these results strongly suggest that the *Drosophila* genome is unmethylated.

**Methylome of Mouse TKO ES Cells.** To further confirm the lack of DNA methylation in *Dnmt2*-only systems we also used bisulfite sequencing to analyze DNA methylation patterns in the mouse TKO ES cell line (Fig. 3A). A detailed analysis of the sequencing data showed that the vast majority (96.5%) of the 75,778,741 genomic cytosine residues that were covered by at least three reads appeared completely unmethylated (ratio <0.1), whereas only 0.03% showed a nonconversion ratio >0.5 (Fig. 3B). A substantially different distribution was observed in a published dataset from wild-type mouse ES cells (33), which showed complete methylation



**Fig. 2.** Characterization of the *Drosophila melanogaster* methylome. (A) Average methylation levels were determined for all cytosine residues and then distributed into bins with increasing methylation ratios (blue bars). For comparison, the corresponding data are also shown for human sperm DNA that was spiked into the *Drosophila* sample before bisulfite conversion (black bars). The actual numerical values of the first bins are 99.7% (*Drosophila*) and 92.9% (human sperm). (B) Dinucleotide sequence context of unconverted cytosines in *Drosophila* (blue) and in human sperm (black). (C) Position-specific nonconversion ratios (red) and coverage (gray) of the *Drosophila* *Invader4* element. Results are shown for the sequence with the lowest conversion rate among genomic *Invader4* elements. The specific region previously reported to be methylated (24) is indicated as a green bar. Sequence position numbers refer to GenBank accession AE014135.3.



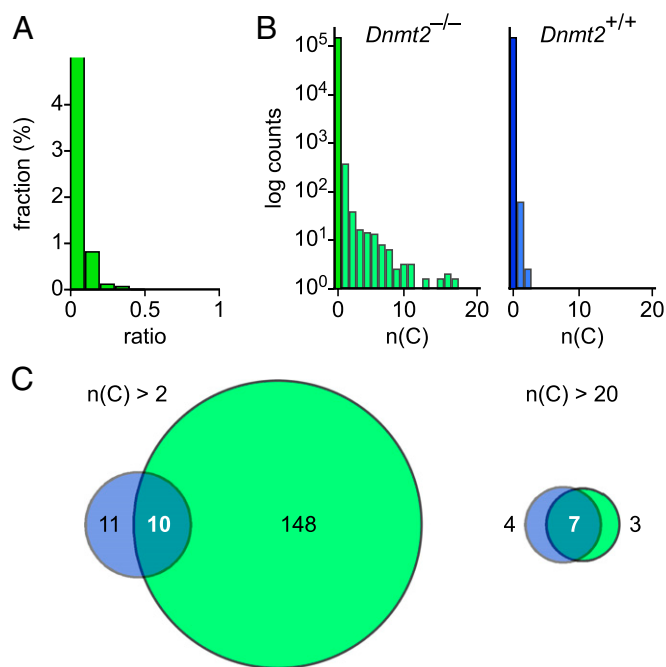
**Fig. 3.** Characterization of DNA methylation in the TKO mouse ES cell model. (A) Schematic illustration of Dnmt genotypes in wild-type and TKO mouse ES cells. (B) Average methylation levels were determined for all cytosine residues and then distributed into bins with increasing methylation ratios (orange bars). For comparison, the corresponding data are also shown for wild-type mouse ES cells (gray bars). The actual numerical values of the first bins are 86.9% (wild type) and 96.5% (TKO). (C) Fractions of nonconverted (ratio >0.1) CpN dinucleotides in wild-type cells (gray bars) and TKO cells (orange bars).

(ratio >0.9) for 2.5% of the cytosine residues (Fig. 3B). A more detailed analysis showed a high proportion of CpG methylation and significant levels of non-CpG methylation in wild-type ES cells (Fig. 3C), consistent with earlier observations (34, 35). Even with a low-stringency cutoff (ratio >0.1), the TKO cells showed substantially reduced signals (Fig. 3C), which suggests that the TKO genome is unmethylated.

**Analysis of Control Datasets.** To further determine the significance of the observed nonconverted cytosine residues, several controls were used (Table 1). (i) An unmethylated PCR fragment that had been spiked into the TKO sample before bisulfite conversion, (ii) human sperm DNA that was spiked into the *Drosophila* sample before bisulfite conversion, and (iii) a *Dnmt2*-deficient *Drosophila* strain. Further data analysis showed that the difference in the conversion efficiencies between the TKO dataset and the spiked-in control was small (0.12%, Table 1), but statistically significant ( $P = 0.012$ , Fisher's exact test). This could be related to an intrinsically higher conversion efficiency of the PCR fragment, relative to genomic DNA. We therefore also analyzed the difference between the *Drosophila* sample and the spiked-in human sperm control. Indeed, the difference in the deamination rates was very small (0.04%, Table 1) and not statistically significant ( $P = 1.0$ , Fisher's exact test). These results provide important confirmation for the absence of defined DNA methylation patterns in *Dnmt2*-only organisms.

Finally, to directly investigate the significance of *Dnmt2* for the remaining nonconverted cytosine residues in *Drosophila*, we generated genome-wide methylation profiles from 0- to 2-h-old homozygous *Dnmt2* mutant embryos. We obtained an average strand-specific genome coverage of 24x and a conversion rate of 99.4% (Table 1). Further data analysis showed that the vast majority (99.0%) of cytosine residues appeared completely unmethylated (Fig. 4A). Notably, nonconverted cytosine residues appeared to be slightly more frequent than in the wild-type *Drosophila* sample (Fig. 4A), which further suggests that the remaining cytosine residues represent bisulfite deamination artifacts, rather than *Dnmt2*-mediated DNA methylation. We also used a sliding window approach to identify sequences with an increased density of nonconverted cytosines. The results showed that the vast majority (>99.9%) of 1-kb windows completely lacked nonconverted cytosines (Fig. 4B). Among the remaining windows,





**Fig. 4.** Characterization of the *Dnmt2* mutant *Drosophila* methylome. (A) Average methylation levels were determined for all cytosine residues that were covered by more than three sequence reads and then distributed into bins with increasing methylation ratios (green bars). The actual numerical value of the first bin is 99.0%. (B) Histograms showing the number of non-converted (ratio >0.5) cytosine residues in 1-kb windows. For comparison, the corresponding data are also shown for the wild-type *Drosophila* methylome (blue). (C) Venn diagram showing overlapping windows with >2 (Left) or >20 (Right) nonconverted cytosine residues in wild-type (blue) and *Dnmt2* mutant (green) embryos.

many contained a single nonconverted cytosine residue (Fig. 4B). Notably, a substantial fraction of windows with more than two nonconverted cytosine residues in wild-type embryos also showed inefficient conversion in *Dnmt2* mutant embryos (Fig. 4C). These regions were often characterized by a high CG content and a low base complexity, which would render them relatively resistant to denaturation during the bisulfite conversion step. These results further argue against genuine DNA methylation in *Drosophila* embryos and provide additional support for the notion that *Dnmt2*-only organisms lack DNA methylation.

## Discussion

The DNA methylation status of *Dnmt2*-only organisms has been a controversial topic for a long time. This may be related to the fact that the reported methylation levels were often close to the detection limits of the various methods that were used for DNA methylation analysis (22). The results from chromatographic analyses (23, 36, 37) may also have been affected by contamination with methylated DNA from other organisms, including bacteria. Similarly, immunological detection approaches of 5-methylcytosine in *Drosophila* embryos (38) could have been affected by low antibody specificity. Also, many previous bisulfite sequencing analyses were limited to isolated genomic loci (23, 24), which made them more susceptible to false positive results. Finally, it is also possible that the conserved catalytic mechanism of *Dnmt2* (11) permits a limited “star activity,” i.e. a low enzymatic activity with relaxed substrate specificity, on DNA substrates. This star activity could be responsible for residual amounts of genuine DNA methylation and might become increased under certain experimental conditions (8, 38). However, because we could not detect

any relevant DNA methylation patterns in our analyses, we would interpret these spurious methylation marks as biological artifacts.

The comprehensive nature of whole-genome bisulfite sequencing datasets allows additional quality control steps during data analysis and permits the identification of false positives with higher sensitivity (25). Also, whole-genome bisulfite sequencing protocols use substantially fewer PCR amplification cycles than locus-specific bisulfite sequencing protocols. This reduces the impact of PCR bias, another prominent source for false positive results in bisulfite sequencing (39). Our whole-genome bisulfite sequencing approach therefore allowed an unbiased characterization of the *Schistosoma* and *Drosophila* methylomes and revealed that both organisms lack defined DNA methylation patterns. Our results thus directly contradict previous reports (23, 24) and establish fundamental differences between *Dnmt2*-dependent and *Dnmt1/3*-dependent methylomes. We cannot presently exclude the possibility that *Dnmt2* expression and/or *Dnmt2*-mediated DNA methylation vary according to unknown environmental cues (22). However, our analyses used the same strains and culture conditions as previously reported (23, 24). Furthermore, our results were derived from common laboratory strains of *S. mansoni* and *D. melanogaster* and can therefore be considered as a reference for future studies.

*Schistosoma* and *Drosophila* are important representatives from a phylogenetically diverse group of organisms that do not encode a canonical DNA methyltransferase enzyme (*Dnmt1* or *Dnmt3*), but have retained a *Dnmt2* gene. Our results suggest that the genomes of these *Dnmt2*-only organisms lack DNA methylation, which would significantly expand the number of eukaryotes with unmethylated genomes. A complete absence of DNA methylation was previously assumed for only a small group of organisms, including *Saccharomyces cerevisiae* and *Caenorhabditis elegans*, as these models apparently have lost all *Dnmt* genes and no experimental evidence for genomic DNA methylation has been obtained.

Importantly, the lack of DNA methylation in a considerable number of eukaryotes also suggests that this modification is not essential for the development of these organisms. This is consistent with the notion that DNA methylation has conserved adaptive functions that might be particularly relevant for lineages that are subject to high adaptive pressure (2, 15, 17). When DNA methylation is lost during the evolutionary history of a genome, other epigenetic mechanisms can be used to ensure the plasticity of gene expression programs. Besides chromatin-based mechanisms and small noncoding RNAs, these might also include the *Dnmt2*-dependent modification of RNAs, which has been implied in a variety of adaptive responses (10, 19, 40, 41).

## Materials and Methods

**DNA Samples.** For *Schistosoma* genomic DNA, adult male worms from the *S. mansoni* Puerto Rican strain were homogenized and DNA was isolated using the Qiagen DNeasy Blood and Tissue kit, according to the manufacturer's instructions. For *Drosophila* genomic DNA, 0- to 2-h embryos were collected from *w<sup>1118</sup>* and *Dnmt2<sup>149</sup>* (24) strains. To extract genomic DNA, embryos were homogenized in 2× proteinase K (PK) buffer [200 mM Tris-HCl, pH 7.5, 25 mM EDTA, pH 8, 300 mM NaCl, 2% (wt/vol) SDS], followed by protein digestion with proteinase K for 2 h at 65 °C. DNA was extracted with an equal volume of phenol-chloroform, followed by ethanol precipitation. Samples were then treated with RNase A for 15 min at 37 °C and genomic DNA was purified by phenol-chloroform extraction. For human sperm DNA, sperm cells were purified and then sonicated. Genomic DNA was purified by double phenol-chloroform extraction and ethanol precipitation. For mouse TKO ES DNA, cells derived from the *Dnmt1<sup>-/-</sup>*; *Dnmt3a<sup>-/-</sup>*; *Dnmt3b<sup>-/-</sup>* clone 19 (28), kindly provided by Masaki Okano (Center for Developmental Biology, RIKEN, Kobe, Japan), were grown under feeder-free conditions on gelatin in complete ES medium (42). Before harvesting, the cells were stained by immunofluorescence against 5-methylcytosine and *Dnmt1* for potential parental cell line contaminations. Genomic DNA was prepared using the Qiagen DNeasy Blood and Tissue kit, according to the manufacturer's instructions. Additional details are provided in *SI Materials and Methods*.

**Accession Numbers.** Sequencing data have been deposited in the Gene Expression Omnibus database under accession nos. GSE39996 (*D. melanogaster*, together with human sperm spike-in), GSE39997 (*S. mansoni*), and GSE42170 (mouse TKO ES cell line, together with M13 PCR fragment spike-in).

**ACKNOWLEDGMENTS.** We thank Stephan Wolf, Andre Leischwitz, and Kristina Tabbada for the Illumina sequencing services; Felix Krueger for bioinformatics support; and Tobias Reber for information technology support. This work was supported by grants from the Biotechnology and

Biological Sciences Research Council (BBSRC), the Medical Research Council, and the Wellcome Trust (to W.R.); the National Institutes of Health (NIH) (5R01GM062534-12 to G.J.H.); and the Deutsche Forschungsgemeinschaft (FOR1082 to M.S. and F.L.). P.M.G. is an NIH trainee on Cold Spring Harbor Laboratory Watson School of Biological Sciences NIH Kirschstein–National Research Service Award predoctoral Award T32 GM065094, a William Randolph Hearst Scholar, and a Leslie Quick Junior Fellow. N.O. is a recipient of a BBSRC Collaborative Awards in Science and Engineering studentship and a Babraham Institute/Cambridge European Trust studentship.

- Mohn F, Schübeler D (2009) Genetics and epigenetics: Stability and plasticity during cellular differentiation. *Trends Genet* 25(3):129–136.
- Feinberg AP (2007) Phenotypic plasticity and the epigenetics of human disease. *Nature* 447(7143):433–440.
- Goll MG, Bestor TH (2005) Eukaryotic cytosine methyltransferases. *Annu Rev Biochem* 74:481–514.
- Law JA, Jacobsen SE (2010) Establishing, maintaining and modifying DNA methylation patterns in plants and animals. *Nat Rev Genet* 11(3):204–220.
- Yoder JA, Bestor TH (1998) A candidate mammalian DNA methyltransferase related to pmt1p of fission yeast. *Hum Mol Genet* 7(2):279–284.
- Okano M, Xie S, Li E (1998) Dnmt2 is not required for *de novo* and maintenance methylation of viral DNA in embryonic stem cells. *Nucleic Acids Res* 26(11):2536–2540.
- Dong A, et al. (2001) Structure of human DNMT2, an enigmatic DNA methyltransferase homolog that displays denaturant-resistant binding to DNA. *Nucleic Acids Res* 29(2):439–448.
- Hermann A, Schmitt S, Jeltsch A (2003) The human Dnmt2 has residual DNA-(cytosine-C5) methyltransferase activity. *J Biol Chem* 278(34):31717–31721.
- Goll MG, et al. (2006) Methylation of tRNA<sup>Asp</sup> by the DNA methyltransferase homolog Dnmt2. *Science* 311(5759):395–398.
- Schaefer M, et al. (2010) RNA methylation by Dnmt2 protects transfer RNAs against stress-induced cleavage. *Genes Dev* 24(15):1590–1595.
- Jurkowski TP, et al. (2008) Human DNMT2 methylates tRNA(Asp) molecules using a DNA methyltransferase-like catalytic mechanism. *RNA* 14(8):1663–1670.
- Sunita S, et al. (2008) Crystal structure of the Escherichia coli 23S rRNA:m5C methyltransferase RlmI (YccW) reveals evolutionary links between RNA modification enzymes. *J Mol Biol* 383(3):652–666.
- Iyer LM, Abhiman S, Aravind L (2011) Natural history of eukaryotic DNA methylation systems. *Prog Mol Biol Transl Sci* 101:25–104.
- Jurkowski TP, Jeltsch A (2011) On the evolutionary origin of eukaryotic DNA methyltransferases and Dnmt2. *PLoS ONE* 6(11):e28104.
- Zemach A, Zilberman D (2010) Evolution of eukaryotic DNA methylation and the pursuit of safer sex. *Curr Biol* 20(17):R780–R785.
- Werren JH, et al.; Nasonia Genome Working Group (2010) Functional and evolutionary insights from the genomes of three parasitoid Nasonia species. *Science* 327(5963):343–348.
- Lyko F, Maleszka R (2011) Insects as innovative models for functional studies of DNA methylation. *Trends Genet* 27(4):127–131.
- Tuorto F, et al. (2012) RNA cytosine methylation by Dnmt2 and NSun2 promotes tRNA stability and protein synthesis. *Nat Struct Mol Biol* 19(9):900–905.
- Becker M, et al. (2012) Pmt1, a Dnmt2 homolog in *Schizosaccharomyces pombe*, mediates tRNA methylation in response to nutrient signaling. *Nucleic Acids Res* 40(22):11648–11658.
- Jeltsch A, Nellen W, Lyko F (2006) Two substrates are better than one: Dual specificities for Dnmt2 methyltransferases. *Trends Biochem Sci* 31(6):306–308.
- Schaefer M, Lyko F (2010) Solving the Dnmt2 enigma. *Chromosoma* 119(1):35–40.
- Krauss V, Reuter G (2011) DNA methylation in *Drosophila*—a critical evaluation. *Prog Mol Biol Transl Sci* 101:177–191.
- Geyer KK, et al. (2011) Cytosine methylation regulates oviposition in the pathogenic blood fluke *Schistosoma mansoni*. *Nat Commun* 2:424.
- Phalke S, et al. (2009) Retrotransposon silencing and telomere integrity in somatic cells of *Drosophila* depends on the cytosine-5 methyltransferase DNMT2. *Nat Genet* 41(6):696–702.
- Lister R, Ecker JR (2009) Finding the fifth base: Genome-wide sequencing of cytosine methylation. *Genome Res* 19(6):959–966.
- Feng S, et al. (2010) Conservation and divergence of methylation patterning in plants and animals. *Proc Natl Acad Sci USA* 107(19):8689–8694.
- Zemach A, McDaniel IE, Silva P, Zilberman D (2010) Genome-wide evolutionary analysis of eukaryotic DNA methylation. *Science* 328(5980):916–919.
- Tsumura A, et al. (2006) Maintenance of self-renewal ability of mouse embryonic stem cells in the absence of DNA methyltransferases Dnmt1, Dnmt3a and Dnmt3b. *Genes Cells* 11(7):805–814.
- Xi Y, Li W (2009) BSMAP: Whole genome bisulfite sequence MAPping program. *BMC Bioinformatics* 10:232.
- Lyko F, et al. (2010) The honey bee epigenomes: Differential methylation of brain DNA in queens and workers. *PLoS Biol* 8(11):e1000506.
- Warnecke PM, et al. (2002) Identification and resolution of artifacts in bisulfite sequencing. *Methods* 27(2):101–107.
- Molaro A, et al. (2011) Sperm methylation profiles reveal features of epigenetic inheritance and evolution in primates. *Cell* 146(6):1029–1041.
- Stadler MB, et al. (2011) DNA-binding factors shape the mouse methylome at distal regulatory regions. *Nature* 480(7378):490–495.
- Ramsahoye BH, et al. (2000) Non-CpG methylation is prevalent in embryonic stem cells and may be mediated by DNA methyltransferase 3a. *Proc Natl Acad Sci USA* 97(10):5237–5242.
- Lister R, et al. (2009) Human DNA methylomes at base resolution show widespread epigenomic differences. *Nature* 462(7271):315–322.
- Gowher H, Leisemann O, Jeltsch A (2000) DNA of *Drosophila melanogaster* contains 5-methylcytosine. *EMBO J* 19(24):6918–6923.
- Lyko F, Ramsahoye BH, Jaenisch R (2000) DNA methylation in *Drosophila melanogaster*. *Nature* 408(6812):538–540.
- Kunert N, Marhold J, Stanke J, Stach D, Lyko F (2003) A Dnmt2-like protein mediates DNA methylation in *Drosophila*. *Development* 130(21):5083–5090.
- Warnecke PM, et al. (1997) Detection and measurement of PCR bias in quantitative methylation analysis of bisulphite-treated DNA. *Nucleic Acids Res* 25(21):4422–4426.
- Thiagarajan D, Dev RR, Khosla S (2011) The DNA methyltransferase Dnmt2 participates in RNA processing during cellular stress. *Epigenetics* 6(1):103–113.
- Durdevic Z, et al. (2013) Efficient RNA virus control in *Drosophila* requires the RNA methyltransferase Dnmt2. *EMBO Rep* 14(3):269–275.
- Ficz G, et al. (2011) Dynamic regulation of 5-hydroxymethylcytosine in mouse ES cells and during differentiation. *Nature* 473(7347):398–402.